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Chlorine Dioxide Gas Sterilization under Square-Wave Conditions

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Experiments were designed to study chlorine dioxide (CD) gas sterilization under square-wave conditions. By using controlled humidity, gas concentration, and temperature at atmospheric pressure, standard biological indicators (BIs) and spore disks of environmental isolates were exposed to CD gas. The sporicidal activity of CD gas was found to be concentration dependent. Prehumidification enhanced the CD activity. The *D* values (time required for 90% inactivation) of *Bacillus subtilis* subsp. *niger* ATCC 9372 BIs were estimated to be 1.5, 2.5, and 4.2 min when exposed to CD concentrations of 30, 15, and 7 mg/liter, respectively, at 23°C and ambient (20 to 40%) relative humidity (RH). Survivor tailings were observed. Prehumidification of BIs to 70 to 75% RH in an environmental chamber for 30 min resulted in a *D* value of 1.6 min after exposure to a concentration of 6 to 7 mg of CD per liter at 23°C and eliminated survivor tailing. Prolonging prehumidification at 70 to 75% RH for up to 16 h did not further improve the inactivation rate. Prehumidification by ultrasonic nebulization was found to be more effective than prehumidification in the environmental chamber, improving the *D* value to 0.55 min at a CD concentration of 6 to 7 mg/liter. Based on the current observations, CD gas is estimated, on a molar concentration basis, to be 1,075 times more potent than ethylene oxide as a sterilant at 30°C. A comparative study showed *B. subtilis* var. *niger* BIs were more resistant than other types of BIs and most of the tested bacterial spores of environmental isolates.

Chlorine dioxide (CD) is a powerful oxidizing and bleaching agent. Liquid CD has been used as a bleaching agent in the textile and paper industries since the 1920s. Liquid CD has also been recognized as bactericidal, viricidal, and sporicidal at a minimum concentration of approximately 0.2 mg/liter (15, 22) and is being increasingly studied today for water treatment, because, unlike chlorine, CD does not result in the formation of trihalomethanes or react with ammonia to form chloramines in water (1, 2). Despite its long history of use, it was not until recently that this compound was demonstrated to be sporicidal in gaseous form (20). The sporicidal activity of CD gas has been confirmed by other investigators (9; U.S. patent 4,504,442, Mar. 1985; U.S. patent 4,681,739, July 1987).

The medical device industry has depended on ethylene oxide (EO) gas for sterile product processing for decades. At present, EO is still the only viable sterilant for many medical products, and it plays a vital role in patient care. However, the urgency of searching for alternate gas sterilants is more acute today than ever because of increasing awareness of toxicity (4) and hypersensitivity issues (6, 7, 10, 14) caused by the residue of EO and its derivatives. The possible relationship between EO and the increased risk of cancer is currently under study by the National Institute for Occupational Safety and Health. As a result, the regulation of EO sterilization processing and the allowable EO level in the working environment is much more strict today than it was 20 years ago, and these regulations are likely to become even more strict in the future.

CD gas and its derivatives, chlorite and chlorate, on the other hand, have relatively low toxicities in humans (11–13, 18, 24). They are not mutagenic (16, 17). Also, CD degasses more readily from processed plastic materials (unpublished data). As a gas sterilant, CD can be used at relatively low concentrations, at room temperature, and at atmospheric pressure. The cost of CD gas sterilization is comparable to that of EO. Although the reaction of CD and the plastic materials and the consequent by-products are not well understood and need to be studied, the available information seems to suggest that CD is an attractive potential replacement for EO in industrial sterilization. However, the available data relevant to the industrial application of CD in sterilization processing are limited.

In a laboratory study with an experimental vessel, we explored the feasibility of CD gas sterilization and report herein the results from a square-wave study, in which the subjects being studied, unlike in the ordinary processing cycle, which involves a precycle gas charge-up and a postcycle degassing time lag, experienced a target gas concentration immediately at the beginning of the cycle and an immediate degassing after the cycle. Under the square-wave and other specified conditions, the effects of gas concentration and prehumidification on CD sporicidal activity, which were too rapid to study under the ordinary sterilization cycle, were accurately assessed. Moreover, we also studied the CD resistance of various types of standard biological indicators (BIs) and spores of environmental isolates in order to identify BIs with specific resistance to CD for use in potential industrial processing validation.

MATERIALS AND METHODS

BIs and bacterial spores. *Bacillus subtilis* subsp. *niger* ATCC 9372 BIs (10^6 spores per BI), with a *D* value (time required for 90% inactivation) of 3.0 min (600 mg of EO per liter, 60% relative humidity [RH], 54°C, and 22 to 23 pounds per square inch gauge), and *Bacillus stearothermophilus* ATCC 12980 BIs, with a *D* value of 2.0 min (121°C saturated steam), were purchased from American Sterilizer Co. (Erie, Pa.). *Bacillus pumilus* ATCC 27142 BIs, with a *D* value of 0.15 Mrads (gamma irradiation), were purchased from North American Science Associates, Inc. (Northwood, Ohio). *B. subtilis* 5230 BIs, with *D* values of 0.41 min (121°C saturated steam) and 52.36 min (121°C dry heat), were prepared by Baxter Healthcare Corp. *Clostridium sporogenes* ATCC 19404 was purchased from the American Type Culture

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Collection (ATCC; Rockville, Md.). All other spores were bioburden or were isolates from the environment and were determined to the species level with a Vitek system (model AMS 120, McDonald Douglas Corp., St. Louis, Mo.).

Since *B. subtilis* subsp. *niger* is recognized as a standard BI for chemical sterilants by the medical device industry, it was used as the standard organism for comparative evaluation.

All spore-forming isolates except *Aspergillus niger* and *C. sporogenes* were sporulated on nutrient agar fortified with 0.02% manganese sulfate at 35°C until at least 90% of the cells were sporulated. *A. niger* was sporulated on Sabouraud dextrose agar and was incubated at 25°C for more than 2 weeks until ascospores were formed. *C. sporogenes* was sporulated on fluid thioglycolate agar at 35°C and was incubated anaerobically.

The spores were collected, filtered through three layers of sterile, 25-μm-pore-size nylon mesh, and then washed extensively by differential centrifugation. The spore stocks in water were stored at 2 to 4°C. Approximately 10^6 spores in 10 μl were inoculated on 1/4-in. (0.6 cm) analytical paper disk blanks (Schleicher & Schuell, Inc., Keene, N.H.), air dried overnight under a laminar hood, and stored in a cool area (22 to 24°C) before testing.

Gas sterilizer. An evacuable glove box (36 in. [91 cm] long by 30 in. [76 cm] in diameter; model HE-135-5 Vac-Lab; Vacuum Atmospheres Corp., Hawthorne, Calif.) equipped with a viewing window and glove ports was used as a gas sterilizer. The vessel can withstand a vacuum to less than 1 mm Hg (133.3 Pa) and pressure to less than 2 pounds per square inch gauge. The main chamber was connected to a sealable antechamber and was equipped with sampling ports and connections for gas, steam, and water. Electric receptacles were available inside the vessel to accommodate equipment, such as an electric fan (model 3-15-2450; Howard Industries, Milford, Ill.) for circulation. A water-circulating radiator (model 1802K1; McMaster Carr, Elmhurst, Ill.) was seated horizontally at the bottom of the vessel to supply heat. The vessel was coated with CD-resistant material before use. The setup of the sterilizer is schematically depicted in Fig. 1.

CD gas generation and measurement. CD gas was generated by passing a 3 or 5% chlorine gas-air mixture through a column containing approximately 500 g of sodium chlorite chips (Olin Chemicals, Stamford, Conn.) by the method described by Hutchison and Mecham (U.S. patent 2,309,457, Jan. 1943) and Woodward et al. (26). The gas was drawn into the evacuated sterilizer and was evenly distributed by the fan. To measure the gas concentration, samples were taken through a sampling port with a septum by using a 10-ml gas-tight syringe (Precision Sampling Corp., Baton Rouge, La.), injected into a solution of potassium iodide, and quantitated by titration with an amperometric titrator (model 821A009U23; Fisher Scientific, Pittsburgh, Pa.) or with a Titralab titrator (model 32; Radiometer, Copenhagen, Denmark) by the method described by Aieta et al. (3).

Sample prehumidification. BIs were prehumidified in a custom-made, 6-ft^3 (0.17-m^3) flexible plastic isolator (Standard Safety Corp., Palatine, Ill.) by using an ultrasonic nebulizer (model 100 HD; DeVilbiss Corp. Somerset, Pa.) at room temperature (23°C) and were monitored with a battery-operated portable humidity indicator (model HMI 31; Vaisala, Helsinki, Finland) or in an environmental chamber (model FRH-251C; Blue M Co., Blue Island, Ill.) at 30°C and monitored with a built-in wet bulb-dry bulb device. For nebulization, sterile distilled water was fed by the nebulizer into the isolator for several minutes, until the RH in the isolator was equilibrated to the desirable level. Both the isolator and the environmental chamber were equipped with glove ports that allowed sample maneuvering. The BIs or spore disks were humidified on a sterile nylon mesh screen under the desired RH and maintained for appropriate time periods. The humidified disks were transferred into sterile, 5-ml, screw-cap vials, which were stored in the chambers with their caps open during the humidification procedure. The vials were sealed with airtight caps and transferred into the sterilizer. When experiments were conducted in dry cycles, the BIs were exposed to ambient RH (20 to 40%) and were monitored with the portable humidity indicator without additional humidification. All BIs and spore samples tested had no packaging, but one set of the BIs in a comparative study for CD gas penetration were contained in standard glycine BI pouches.

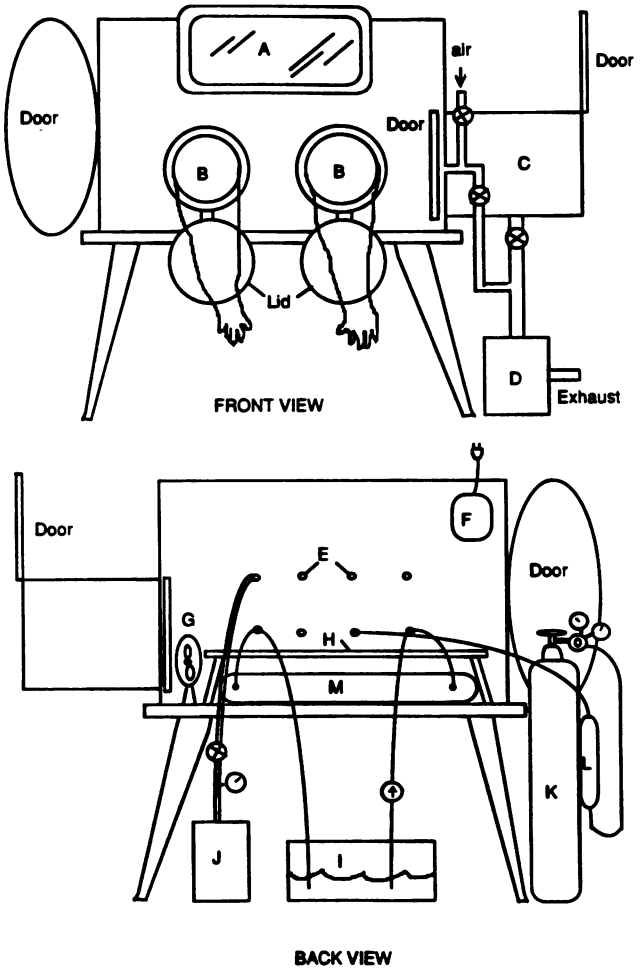

FIG. 1. Schematic depiction of the experimental sterilizer used in CD gas sterilization studies. Descriptions of letters: A, viewing window; B, glove ports; C, antechamber; D, vacuum pump; E, ports for gas, steam, air, hot water, and gas sampling; F, electric receptacle for a circulating fan; G, electric fan; H, nylon mesh screen; I, hot water bath; J, steamer; K, compressed 3 or 5% chlorine-air mixture cylinder; L, sodium chlorite column; M, water radiator.

Square-wave sterilization cycle. All studies were carried out at atmospheric pressure. To prepare the cycle, the sterilizer was evacuated to approximately 25 mm Hg (3,332.5 Pa). As predetermined by testing, an appropriate amount of

the chlorine gas-air mixture was charged into the evacuated sterilizer through the sodium chlorite column, which converted the chlorine gas to CD with approximately 93 to 95% efficiency. The vessel was brought up to atmospheric pressure by the addition of room air.

When humidity was used, steam produced by a small pressure steamer (Parr Instruments, Moline, Iowa) was charged into the sterilizer to a level that was predetermined by testing before the addition of CD. All sealed vials containing BIs and spore samples were opened only after the desirable RH, CD concentration, temperature, and vessel pressure were achieved and stabilized.

The cycle began when the BIs or spore disks were removed at 1-min intervals from the individual vial with a pair of forceps and dropped onto a sterile nylon mesh screen for exposure. Replicates of seven disks per exposure time were tested. At the end of exposure, each disk was dropped into an individual screw-cap test tube containing 10 ml of sterile 0.02% sodium thiosulfate solution, which completely neutralized the action of CD. The thiosulfate tubes were closed and stored in the sterilizer during equilibration and were recapped immediately on the addition of disks until all samples were processed. The sterilizer was then degassed and flushed twice with air before all samples were withdrawn for bioassay. Control BIs were dropped into the thiosulfate tubes and sealed before the cycle operation, and the sealed tubes were exposed to CD in a manner identical to that of the other samples in the vessel.

Microbial assay. The BIs or spore disks were macerated by sterile cell homogenizers. The homogenized materials were ultrasonicated in a sonicator (model 32; Bransonic Cleaning Instrument, Shelton, Conn.) by the method described by Jeng et al. (D. K. Jeng, L. I. Lin, and L. V. Hervey, J. Appl. Bacteriol. [United Kingdom], in press). A 1-ml portion or the entire volume of each bacterial spore sample was then bioassayed by a standard pour plate method by using Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) or fluid thioglycolate agar medium. *A. niger* was similarly assayed in Sabouraud dextrose agar medium fortified with 0.8 μg of dichloran per ml (D. K. Jeng and N. Weber, Abstr. Annu. Meet. Soc. Indust. Microbiol., SIM News **36**:A-22, 1986). The plates were incubated aerobically or anaerobically at 23 or 35°C for 3 or 7 days before enumeration. Prolonged incubation did not result in an increase in the colony numbers.

RESULTS

Effect of gas concentration. The effect of gas concentration on the sporicidal activity of CD against *B. subtilis* subsp. *niger* was studied in ambient humidity (20 to 40%) (dry cycle) at room temperature (23°C). Figure 2 shows that the sporicidal kinetics of CD were concentration dependent, with a higher gas concentration resulting in faster killing. The shoulders of the initial curves indicate a time lag for sporicidal activity that was presumably due to the time required for gas diffusion. In dry cycles, spore survivor tailing was commonly observed. The *D* values were estimated to be 1.5, 2.5, and 4.2 min from the straight portion of the inactivation curves for gas concentrations of 30, 15, and 7 mg/liter, respectively.

Effect of glycine pouch material. Seven replicates of prehumidified (60 to 65% RH at 23°C in the environmental chamber) *B. subtilis* subsp. *niger* BIs in glycine pouches were compared with the same BIs without packaging in 9 to 10 mg of CD gas per liter and 60 to 65% RH at 30°C. Figure 3 shows that there was an initial delay (about 3 to 5 min) in the sporicidal activity of the BIs contained in glycine pouches, presumably because of the time requirement for gas diffusion. The difference in spore inactivation between the packaged and unpackaged samples was minimized at later exposure times. There was little difference in the estimated *D* values between these samples. The *D* value was estimated to be 2.4 min.

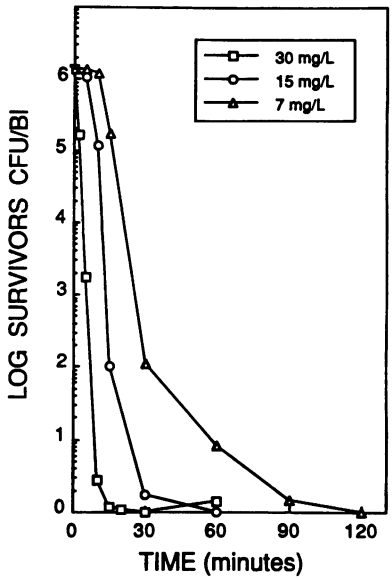

FIG. 2. Sporicidal kinetics of CD gas sterilization in ambient humidity. *B. subtilis* subsp. *niger* BIs were exposed to 7, 15, or 30 mg of CD gas per liter under square-wave conditions at 23°C. Each of the datum points represents an average of seven replicates of BIs.

Effect of prehumidification. *B. subtilis* subsp. *niger* BIs were prehumidified in an environmental chamber to 70 to 75% RH at 30°C or by nebulization in the isolator to 70 to 75% RH at 23°C for appropriate time periods and then

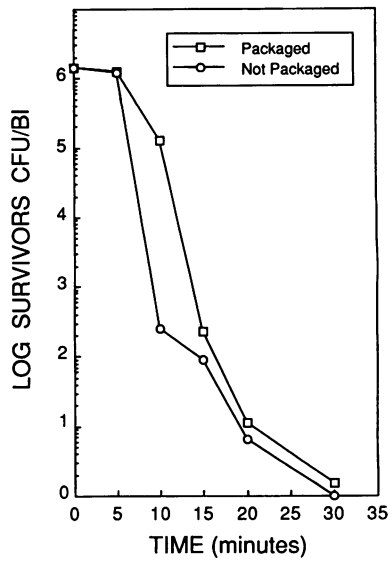

FIG. 3. CD gas penetration through glycine paper pouch under square-wave sterilization conditions. Each of the datum points represents an average of seven replicates of samples.

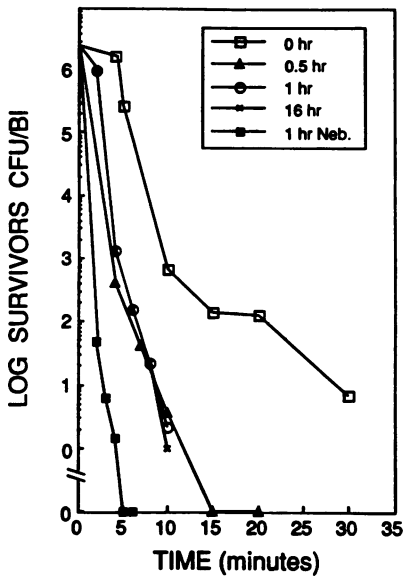

FIG. 4. Effect of prehumidification on CD sporicidal activity. *B. subtilis* subsp. *niger* BIs were prehumidified as described in the text and exposed to 6 to 7 mg of CD per liter at 70 to 75% RH and 23°C under atmospheric pressure. The 0 below the lines that break the ordinate represents a true 0 point on a linear scale. Each of the datum points represents an average of seven replicates of BIs.

exposed to 6 to 7 mg of CD gas per liter (70 to 75% RH, 23°C). The dry BIs that were not prehumidified were exposed under the same conditions for purposes of comparison. Figure 4 shows the effect of humidity on CD gas sporicidal activity with *B. subtilis* subsp. *niger* BIs. The viability of the dry BIs was reduced gradually and had a typical survivor tailing that could not be eliminated during the humidified exposure cycle. The residual spores were not totally killed after 30 min of exposure. On the other hand, the samples that were prehumidified in 70 to 75% RH for 0.5, 1, and 16 h had much faster but similar killing rates. All spores were eliminated in 15 min in all cases, with an estimated *D* value of 1.6 min. No tailing was observed. The CD gas sporicidal curve was significantly sharper for BI samples that were humidified with the ultrasonic nebulizer to 70 to 75% RH for 1 h, inactivating 10^6 spores in 5 min without tailing with an estimated *D* value of 0.55 min.

Resistance of BIs and environmental spores against CD. To provide a comparison with *B. subtilis* subsp. *niger* BIs, alternate BIs and environmental spores on disks containing approximately 10^6 spores per disk were prehumidified to 70 to 75% RH for 1 h in an environmental chamber at 30°C and then exposed to 6 to 7 mg of CD gas per liter in 70 to 75% RH at 30°C for 4 min under square-wave conditions. The 4-min exposure time was chosen to be in the mid-range of the BSN inactivation curve. Table 1 shows the relative resistance of the average of seven replicates of each type of BI and spore sample compared with that of *B. subtilis* subsp. *niger*. Among the tested bacterial BIs, only *B. stearothermophilus* showed similar resistance to *B. subtilis* subsp. *niger*. In the spore samples tested, one strain of *Bacillus polymyxa* was more than twice as resistant as the standard *B. subtilis* subsp. *niger* isolate. This particular strain of *B. polymyxa* excreted a large amount of viscous material which was difficult to remove by extensive washing procedures. The extracellular material may have been responsible for the resistance. The fungal spores of *A. niger* were significantly more resistant than were those of *B. subtilis* subsp. *niger*. All other bacterial spores were more sensitive to the CD gas than were those of *B. subtilis* subsp. *niger*.

TABLE 1. Relative resistance of BIs and spores of environmental isolates against CD gas

BIs or spore disks	Relative resistance (%)[a]
BIs	
B. subtilis subsp. *niger* ATCC 9372	100.00
B. stearothermophilus ATCC 12980	100.89
B. pumilis ATCC 27142	81.54
B. subtilis 5230	67.71
Spore disks	
B. polymyxa	262.65
A. niger	158.33
B. megaterium	84.43
B. subtilis subsp. *niger*	73.51
B. subtilis	68.83
B. megaterium	67.50
B. polymyxa	66.03
Bacillus sp.	57.98
B. subtilis	34.88
B. pumilus	30.75
Bacillus sp.	28.88
C. sporogenes ATCC 19404	0.00
B. coagulans	0.00
B. subtilis subsp. *niger*	0.00
B. pumilus	0.00

[a] Seven replicates of BIs or spore disks were prehumidified and exposed to CD gas as described in the text. The relative resistance was calculated based on the reciprocal of the slopes of the CD spore inactivation lines (exposure time/$\log_{10}$ spore reduction) in comparison with that of the standard *B. subtilis* subsp. *niger* BI, which was arbitrarily set to 100%.

DISCUSSION

The experimental design described here, with the help of the described equipment, allowed us to study CD sporicidal kinetics under square-wave conditions with relatively short exposure times. Under the square-wave conditions, with controlled RH, gas concentration, temperature, and pressure, the effect of gas concentration in ambient humidity, the effect of glycine paper on gas penetration, the effect of prehumidification time, and the method of humidification were studied. The CD gas resistances of alternate BIs and environmental spores versus that of *B. subtilis* subsp. *niger* were compared.

In this study we confirmed the observation that prehumidification is important to CD sporicidal activities (9; U.S. patent 4,504,442, 1985; U.S. patent 4,681,739, 1987). The CD cycle performed in ambient humidity reduced almost 4 log units of *B. subtilis* subsp. *niger* spores in 15 min but seemed unable to eliminate the remaining residual survivors even after prolonged exposure. Prehumidification, even at room temperature (23°C), greatly enhanced the effectiveness of CD sterilization, especially in eliminating the survivor tailings. As little as 30 min of prehumidification was sufficient to produce the same effect as that at 16 h of treatment. Prolonged humidification was not necessary for sterilizing unpackaged BIs.

CD penetrated the glycine paper pouch without difficulty. A short initial lag in lethality did not affect the overall *B. subtilis* subsp. *niger D* value. In a conventional processing cycle with an evacuation step, the penetration would be improved further. In a separate study under a 30-min CD

sterilization cycle with 30 mg of CD per liter at 75 to 80% RH and 23°C with preevacuation and poststerilization air-flushing steps (two flushings), we have found that CD can penetrate through sealed polyvinyl chloride tubes of various sizes with a wall thickness of 0.04 to 0.05 mm and sterilize the tube interiors (unpublished data). The gas also penetrated various sizes of rigid polyvinyl chloride medical device containers (thickness, approximately 0.03 mm) with Tyvek covers and sterilized the BIs in the containers (unpublished data). CD could also be degassed from Tyvek packaged products in a matter of a few hours in an atmospheric environment to a below-detectable level, as measured by a continuous gas monitor (MDA Scientific, Lincolnshire, Ill.) with an instrument sensitivity of less than 10 ppb (v/v) (unpublished data).

The samples that were prehumidified by nebulization showed a greater effect on CD inactivation than did those that were prehumidified in the environmental chamber. The ultrasonic nebulizer that was operated at 1.7 MHz produced microdroplets in a range from 0.5 to 8 μm, with a mean size of 5 μm. These droplets would be much larger than the water molecules equilibrated in the environmental chamber by evaporation. It is likely that the BI disks that were humidified by nebulization absorbed and localized the initial assault of nebulized microdroplets that were not in equilibrium with the environmental RH during the prehumidification time period. This localized and concentrated moisture in the disks could not be detected by the measuring system.

Furthermore, CD was highly soluble in water. At 25°C, under 1 atm of pressure, it was about 23 times as concentrated in aqueous solution as it was in the gas phase with which it was in equilibrium (23, 25). It is likely, therefore, that the faster killing effect observed in the nebulized samples is the direct effect of the localized concentrated CD resulting from a higher water activity in the sample disks.

The square-wave studies allowed us, for the first time, to compare, on a molar concentration basis, the potency of CD and EO as sterilants. The *B. subtilis* subsp. *niger* BIs used in this study had a *D* value of approximately 3.0 min at an EO gas concentration of 600 mg/liter and at 60% RH and 54°C, as measured in a near-square-wave cycle. The *D* value of the same lot of BIs at a CD gas concentration of 9 to 10 mg/liter and at 60 to 65% RH and 30°C was approximately 2.4 min (Fig. 3). Based on the *D* values given above, the gas molar concentration multiplied by the time for 90% inactivation (21) of EO was calculated to be about 0.9164, versus a value of 0.008 for CD. CD is therefore almost 115 times more active than EO as a sterilant under the processing conditions described here. The temperature coefficient, Q_{10}, of EO is 3.2 at a processing concentration of 440 mg/liter and 2.3 at 880 mg/liter (8). Intrapolated from these figures, the Q_{10} of EO at 600 mg/liter should be in the neighborhood of 2.86. If both EO and CD were compared at the same processing temperature of 30°C and under a similar processing RH, the sporicidal activity of CD is estimated to be more than 1,075 times more potent than EO. The rapid action of CD may relate to its oxidative mode of action, whereby it can attack the cell surface membrane proteins, including enzymes for propagation. The CD oxidative reaction with proteins is a rapid process that is sometimes too rapid to measure (19). EO gas, with its principal biochemical effect by alkylation of the nucleophilic groups of nucleic acids (5), must penetrate to the deeper sites in spores to reach the primary lethal targets.

The present study showed that *B. subtilis* var. *niger* ATCC 9372 is more resistant than most of the bacterial BIs and spores of the environmental isolates tested except for *B. polymyxa*. *B. polymyxa* formed a layer of extremely viscous extracellular material when it was grown on sporulation medium. The extracellular material was difficult to remove by the washing procedure. It is possible that the residues of this material may form a protective coating on spores or may serve as a binding site for moisture or CD gas. Regardless of the mechanisms of resistance, this observation calls for caution and emphasis on bioburden analysis for industrial processing.

In addition to this strain of *B. polymyxa*, the fungal ascospores of *A. niger* were significantly more resistant than *B. subtilis* subsp. *niger*. Ascospores of *A. niger* have been found to be more resistant than some standard BIs to certain chemical and physical sterilants. It is not known whether the larger spore size, which forms larger clumps of the spore inoculum in the disks, would have affected the sterilization efficacy. In a practical sense, however, *B. subtilis* var. *niger* ATCC 9372 BI is a good choice as a biological indicator for CD processing.

CD gas can effectively sterilize BIs and other spore disks under defined conditions. The effectiveness of CD in sterilizing certain medical devices has been evaluated in this laboratory. A limited toxicological study of the residues of CD and its derivatives extracted from exposed medical devices has also been conducted. The results will be reported elsewhere.

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